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(54) PROCESSUS DE TOLERANCE AU SEL PAR LES PLANTES DE GRANDE CULTURE
(54) ENGINEERING SALT TOLERANCE IN CROP PLANTS

(57)

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Hydropathy plot of AtNHX6 amino acid sequence. (Kyte and Doolittle hydrophobicity values using a window size of ten amino acids.)

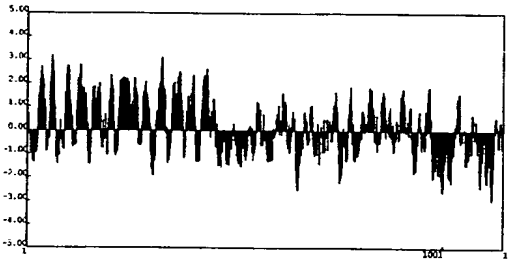

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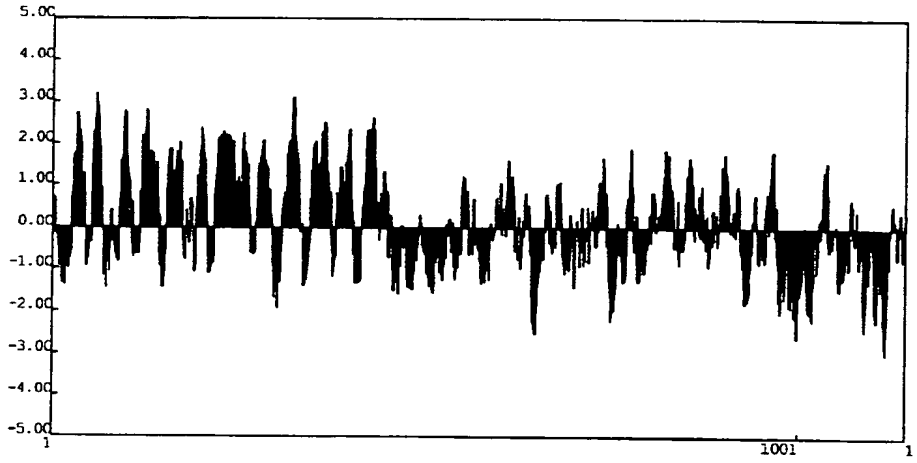
Figure 2. Hydropathy plot of AtNHX6 amino acid sequence. (Kyte and Doolittle hydrophobicity values using a window size of ten amino acids.)



(57) Abrégé/Abstract:
The invention is an isolated nucleic acid molecule encoding a Na^{+}/H^{+} exchanger protein for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance. In a preferred embodiment, the nucleic acid is obtained from Arabidopsis thaliana. Crop species transformed with the gene are capable of surviving in soil with high salt levels that would normally inhibit growth of the crop species.

Abstract

The invention is an isolated nucleic acid molecule encoding a Na^+/H^+ exchanger protein for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance. In a preferred embodiment, the nucleic acid is obtained from *Arabidopsis thaliana*. Crop species transformed with the gene are capable of surviving in soil with high salt levels that would normally inhibit growth of the crop species.

TITLE: ENGINEERING SALT TOLERANCE IN PLANTS

BACKGROUND OF THE INVENTION

Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, which are predominantly sensitive to the presence of high concentrations of salts in the soil. Large terrestrial areas of the world are affected by levels of salt inimical to plant growth. It is estimated that 35-45% of the 279 million hectares of land under irrigation is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands, (which comprise 25% of the total land of our planet). Salinity has been an important factor in human history and in the life spans of agricultural systems. Salt impinging on agricultural soils has created instability and has frequently destroyed ancient and recent agrarian societies. The Sumerian culture faded as a power in the ancient world due to salt accumulation in the valleys of the Euphrates and Tigris rivers. Large areas of the Indian subcontinent have been rendered unproductive through salt accumulation and poor irrigation practices. In this century, other areas, including vast regions of Australia, Europe, southwest USA, the Canadian prairies and others have seen considerable declines in crop productivity.

Although there is engineering technology available to combat this problem, though drainage and supply of high quality water, these measures are extremely costly. In most of the cases, due to the increased need for extensive agriculture, neither improved irrigation efficiency nor the installation of drainage systems is applicable. Moreover, in the arid and semi-arid regions of the world water evaporation exceeds precipitation. These soils are inherently high in salt and require vast amounts of irrigation to become productive. Since irrigation water contains dissolved salts and minerals, an application of water is also an application of salt that compounds the salinity problem.

Increasing emphasis is being given to modify plants to fit the restrictive growing conditions imposed by salinity. If economically important crops could be

manipulated and made salt resistant, this land could be farmed resulting in greater sales of seed and greater yield of useful crops. Conventional breeding for salt tolerance has been attempted for a long time. These breeding practices have been based mainly on the following strategies: a) the use of wide crosses between crop plants and their more salt-tolerant wild relatives [1], b) screening and selecting for variation within a particular phenotype [2], c) designing new phenotypes through recurrent selection [3]. The lack of success in generating tolerant varieties (given the low number of varieties released and their limited salt tolerance) [4] would suggest that conventional breeding practices are not enough and that in order to succeed a breeding program should include the engineering of transgenic crops [5]. Several biochemical pathways associated with stress tolerance have been characterized in different plants and a few of the genes involved in these processes have been identified and in some cases the possible role of proteins has been investigated in transgenic/overexpression experiments. Several compatible solutes have been proposed to play a role in osmoregulation under stress. Such compatible solutes, including carbohydrates [6], amino acids [7] and quaternary N-compounds [8] have been shown to increase osmoregulation under stress. Also, proteins that are normally expressed during seed maturation (LEAs, Late Embriogenesis Abundant proteins) have been suggested to play a role in water retention and in the protection of other proteins during stress. The overexpression of LEA in rice provided a moderate benefit to the plants during water stress [9,10]. A single gene (sod2) coding for a Na^+/H^+ antiport has been shown to confer sodium tolerance in fission yeast [11,12], although the role of this plasma membrane-bound protein appears to be only limited to yeast. One of the main disadvantages of using this gene for transformation of plants is associated with the typical problems encountered in heterologous gene expression, i.e. incorrect folding of the gene product, targeting of the protein to the target membrane and regulation of the protein function.

Plants that tolerate and grow in saline environments have high intracellular salt levels. A major component of the osmotic adjustment in these cells is accomplished by ion uptake. The utilization of inorganic ions for osmotic

adjustment suggests that salt-tolerant plants must be able to tolerate high levels of salts within their cells. However, enzymes extracted from these plants show high sensitivity to salt. The sensitivity of the cytosolic enzymes to salt would suggest that the maintenance of low cytosolic sodium concentration, either by compartmentation in cell organelles or by exclusion through the plasma membrane, must be necessary if the enzymes in the cell are to be protected from the inimical effects of salt.

Plant cells are structurally well suited to the compartmentation of ions. Large membrane-bound vacuoles are the site for a considerable amount of sequestration of ions and other osmotically active substances. A comparison of ion distribution in cells and tissues of various plant species indicates that a primary characteristic of salt tolerant plants is their ability to exclude sodium out of the cell and to take up sodium and to sequester it in the cell vacuoles. Transport mechanisms could actively move ions into the vacuole, removing the potentially harmful ions from the cytosol. These ions, in turn, could act as an osmoticum within the vacuole, which would then be responsible for maintaining water flow into the cell. Thus, at the cellular level both specific transport systems for sodium accumulation in the vacuole and sodium extrusion out of the cell are correlated with salt tolerance.

SUMMARY OF THE INVENTION

We have isolated a gene from *Arabidopsis thaliana* that encodes an Na^+/H^+ antiport, said gene being the subject of the present invention. The gene is useful for preparing salt tolerant cells and plants. The gene product (protein) will allow the extrusion of sodium ions from the cytosol into the extracellular space or apoplast, or into intracellular compartments, such as the vacuole. This gene will allow the engineering of salt tolerant plants by transformation of salt-sensitive crops overexpressing this gene under the control of constitutively active promoters or under the control of conditionally-induced promoters. Plant Na^+/H^+ antiport is also clearly demonstrated by the functional complementation in yeast. Agrobacterium tumefaciens-mediated transformation or particle-bombardment-

mediated transformation will be used depending upon the plant species. Salt tolerance genes isolated from other salt tolerant plants are also used to transform plant cells.

The genes of the invention will also allow the identification of homologous genes from salt tolerant plant species and their use in genetically engineering salt tolerant plants of agricultural and commercial interest.

The genes and the proteins are used in a method for protecting a plant from the adverse affects of a saline environment by incorporating a gene for salt tolerance and/or the protein of the invention into a plant.

The genes may be isolated as described below or synthesized according to techniques known in the art. A protein may be produced from an isolated gene which was isolated using techniques known in the art or synthesized according to techniques known in the art.

The invention relates to an isolated nucleic acid molecule encoding a protein for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance.

The nucleic acid molecule preferably comprises the nucleotide sequence in figure 1 more preferably base pairs 295 to 3473 of figure 1. The nucleic acid molecule may be DNA or RNA. The nucleic acid molecule may be used to transform a cell selected from the group consisting of a plant cell, a yeast cell and a bacterial cell. The sodium ions are extruded from the cell or into an intracellular compartment. The nucleic acid molecule encodes a Na^+/H^+ exchanger protein.

In a preferred embodiment, the nucleic acid molecule is isolated from *Arabidopsis thaliana*. The invention includes an isolated nucleic acid molecule, comprising the DNA sequence in figure 1. The invention also relates to an isolated nucleic acid molecule, comprising a sequence having greater than 35% similarity to the sequences of the invention described in the preceding paragraphs.

In an alternate embodiment, the nucleic acid molecule consists of a sequence selected from the group consisting of 8 to 10 nucleotides of the nucleic

acid molecules of the invention, 11 to 25 nucleotides of the nucleic acid molecule and 26 to 50 nucleotides of the nucleic acid molecule. These nucleic acid molecules hybridize to a nucleic acid molecule described in the preceding paragraphs.

The nucleic acid molecule of the invention may have a sense or an antisense sequence. In another embodiment, the invention is an isolated oligonucleotide consisting of a sequence selected from the group consisting of

RT-PCR:
X6F3-5'-CATGAGAGTCAGTTTCTCAC-3'
X6R3-5'-ATGCTGCCGAAATGCTGAG-3'

RACE:
X65RACE-5'-CAGGCCAGCTCATGAGACCTCTGTGTTCTTTACG-3'
X63RACE-5'-GGAGCATTTGGAGCTGATAGGACTCTACATCG-3'

CLONING INTO YEAST EXPRESSION VECTOR pYPGE15:
X6YF-5'-GCCGCCCGGGATGACGACTGTAATCGAC-3'
X6YR-5'-CCGGCGTCGACTCATAGATCGTTCCTGAAAACG-3'

CLONING AtNHX6 INTO pBISN1 (OVEREXPRESSION CONSTRUCT):
X6AF-5'-CGCGGTCGACATGACGACTGTAATCGACGCGACGATG-3'
X6AR-5'-GCCGCCCGGGTCATAGATCGTTCCTGAAAACG-3'

CLONING INTO pGEX2TK (GST-FUSION CONSTRUCT):
X6GSTF-5'-CGCCGGATCCGCAATCATCTTCAACATTGG-3'
X6GSTR-5'-CGGCGAATTCTAGATCGTTCCTGAAAACG-3'

The invention includes an isolated oligonucleotide consisting of 5 to 15 nucleotides of these oligonucleotides. The invention includes an isolated

oligonucleotide consisting of a sequence homologous to the oligonucleotides of the invention.

In an alternate embodiment, the invention is an expression vector comprising a nucleic acid molecule of the invention. The expression vector preferably consists of a promoter selected from the group consisting of a super promoter, a 35S promoter of cauliflower mosaic virus, a drought-inducible promoter, an ABA-inducible promoter, a heat shock-inducible promoter, a salt-inducible promoter, a copper-inducible promoter, a steroid-inducible promoter and a tissue-specific promoter.

The invention is a protein produced from the nucleic acid molecules of the invention. The invention is also a protein produced from the expression vector. The protein is used for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance.

In a preferred embodiment, the protein has the amino acid sequence in figure 1 more preferably base pairs 295 to 3473 of Figure 1. The proteins may be homologous to the protein in figure 1. In an alternate embodiment, the proteins comprise a sequence having greater than 50% identity to the protein in figure 1. The proteins are Na^+/H^+ exchanger proteins.

The proteins are preferably isolated isolated from *Arabidopsis thaliana*.

The invention includes peptides consisting of at least 5 amino acids of the proteins described in the preceding paragraphs. In another embodiment, the peptides consist of 41 to 75 amino acids of the proteins described in the preceeding paragraphs.

The invention also includes isolated nucleic acid molecules encoding the proteins of the invention. The isolated nucleic acid molecule preferably encodes the protein of figure 1b.

The invention also includes a monoclonal antibody or polyclonal antibody directed against a protein of the invention.

Another embodiment of the invention includes a transformed microorganism comprising an isolated nucleic acid molecule of the invention.

The invention also includes a transformed microorganism including an expression vector.

The invention includes a plant cell transformed with a nucleic acid molecule of the invention. The invention also includes a yeast cell transformed with the nucleic acid molecule of the invention. In another embodiment, the invention is a plant, plant part or seed, generated from a plant cell transformed with a nucleic acid molecule of the invention. The invention also relates to a plant, plant part, seed or plant cell transfected with a nucleic acid molecule of the invention. The plant, plant part, seed or plant cell is preferably selected from a species selected from the group consisting of potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, sorghum, alfalfa and salicornia. The invention also includes a method for producing a peptide of the invention by culturing a plant, plant part, seed or plant cell of the invention and recovering the expressed peptide from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

A preferred embodiment of the invention is described in relation to the drawings in which:

Figure 1. Isolated cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana*.

(a) sequence for AtNHX6.
(b) Amino acid sequence for AtNHX6.

Figure 2. Hydropathy plot of AtNHX6 amino acid sequence.

Figure 3. Alignment of the predicted amino acid sequence of Arabidopsis AtNHX6 with Na+/H+ antiports from other organisms. Sequences were aligned using the Clustal W program [19]. Sequences and GeneBank accession numbers are: Synec—Synechocystis, BAA17925, NhaG—Bacillus subtilis,

BAA89487, NAH1— Homo Sapiens, P19634, NHE2A—Homo Sapiens, AAD41635, NAH1M– Mus musculus, Q61165.

Figure 4. A Southern blot of Arabidopsis genomic DNA. Genomic DNA (10μg per lane) was digested with various restriction enzymes, separated on a 1.0% agarose gel, transferred onto a GeneScreen Plus membrane (Amersham), and hybridized to a radiolabelled AtNHX6 cDNA as described in Materials and Methods. Restriction enzymes used were: KpnI, SmaI, XbaI, PstI, BamHI, and EcoRI.

Figure 5. RNA blot of AtNHX6 expression in different tissues. Total RNA (35μg) was separated on a 1.2% formaldehyde agarose gel, transferred to a GeneScreen Plus membrane (Amersham) and hybridized to a radiolabeled EcoRI fragment of the AtNHX6 cDNA probe as described in Materials and Methods. Tissues in each lane were as follows: 1, inflorescence stem; 2, flower (including sepals); 3, seedling shoot; 4, root; 5, mature leaf.

Figure 6. Yeast complementation. Three dilutions of each colony culture were spotted on APG plates each with different pH values and NaCl concentrations and grown for 2-4 days at 30°C. Four different pH values of 4.5, 5.5, 6.5 and 7.5 were used. For each pH value the following NaCl concentrations were used: 0, 25mM, 50mM, 75mM, 100mM and 200mM . The Δena1-4 Δnhx_1 yeast mutant expressing AtNHX6 showed enhanced growth at pH 4.5 at almost all NaCl concentrations (only the control and 50mM NaCl plates are shown) compared to mutant yeast carrying the empty pYPGE15 plasmid. Under these conditions, the plant AtNHX6 functionally complements the Δena1-4 Δnhx1 mutation.

DETAILED DESCRIPTION OF THE INVENTION

We have isolated a cDNA encoding a unique Na^+/H^+ exchanger from *Arabidopsis thaliana*. The cDNA sequence and the corresponding predicted amino acid sequence for AtNHX are presented in Figure 1. The longest open reading frame of 3438 bp encodes a protein of 1146 amino acids with a predicted MW of about 127 kDa. A comparison of this full length cDNA with the *Arabidopsis* genome sequence (AC006532) revealed the presence of 22 introns and 23 exons. This protein encoded by the open reading frame was DIFFERENT FROM THE sequence predicted by the Arabidopsis genomic sequence (AAD20091). This sequence encodes the full length exchanger given that the cDNA region immediately upstream of the start codon contains predicted stop codons in all three reading frames. In addition, a transcript of approximately 3.6 kb, which corresponds roughly in size to the predicted mRNA for AtNHX6, was observed on RNA blots. Based on the amino acid sequence of AtNHX6, 12 transmembrane domains are predicted and a relatively hydrophilic C-terminal region is also predicted (Figure 2). AtNHX6 shows some similarity at the amino level to Na^+/H^+ exchangers isolated from a variety of organisms (Figure 3).

Southern Blot Analysis (Figure 4) suggests that AtNHX6 is likely present as a single copy gene in *Arabidopsis*. A Northern blot (Figure 5) showed that AtNHX6 was expressed most abundantly in flower tissues, moderately in shoot, leaf and stem tissues, and only relatively weakly in root tissues.

This is not the first isolation of a gene encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana*. It is widely known amongst those skilled in the art that *Arabidopsis thaliana* serves as a model plant for many plant species.

Many genes identified in *Arabidopsis thaliana* have striking DNA sequence similarity to genes encoding the homologous protein in other plant species. Using the techniques described herein and others known to those skilled in the art, it will be obvious that the gene encoding the homologous Na^+/H^+ exchanger in other plant species including, but not limited to plants of agricultural and commercial interest, will have DNA sequence identity >35% to the DNA sequence shown in figure 1. Some plants species may have DNA with an identity >50%, >60%, >70%, >80% or >90% to the DNA sequence in figure 1.

The proteins encoded by the homologous Na^+/H^+ exchanger gene in other plant species will have amino acid sequence similarity >50% to the amino acid sequence shown in figure 1. Some plants species may have proteins with a sequence similarity >60%, >70%, >80% or >90% to the amino acid sequence in figure 1. Identity and similarity can be determined by blast II (the reference for BLAST2SEQUENCES is Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250; see also http://www.ncbi.nlm.nih.gov/gorf/bl2.html.

The proteins of the invention allow the extrusion of sodium ions from the cytosol, either through the accumulation of sodium ions into the vacuoles or into the extracellular space, thus providing the most important trait for salt tolerance in plants. The genes of the invention allow the engineering of salt tolerant plants by transformation of salt-sensitive crops overexpressing this gene under the control of constitutively active promoters or under the control of conditionally-induced promoters.

Those skilled in the art will recognize that the nucleic acid molecule sequence in figure 1 is not the only sequence which may be used to provide salt tolerance in plants. The genetic code is degenerate so other nucleic acid molecules which encode a protein identical to the amino acid sequence in figure1 may also be used. The sequence of the other nucleic acid molecules of this invention may also be varied without changing the protein encoded by the sequence.

The invention also includes genes with mutations that cause an amino acid change in a portion of the protein not involved in providing salt tolerance or an amino acid change in a portion of the protein involved in providing salt tolerance so that the mutation increases or decreases the activity of the protein.

The nucleic acid molecules of the invention (including fragments of the molecules) can be used to construct probes to detect nucleotide sequences according to techniques known in the art. The probes may be used to detect genes that encode proteins similar to the proteins of the invention. For example,

a probe having at least 10 bases will hybridize to similar sequences under stringent hybridization conditions (Sambrook et al. 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor). A probe having at least 15 bases may be used in another embodiment.

Changes in the nucleotide sequence which result in production of a chemically equivalent or chemically similar amino acid are included within the scope of the invention. Variants of the proteins of the invention may be made, for example, with protein engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine.

Peptides comprising one or more D-amino acids are contemplated within the invention. Also contemplated are peptides where one or more amino acids are acetylated at the N-terminus. Those of skill in the art recognize that a variety of techniques are available for constructing peptide mimetics with the same or similar desired biological activity as the corresponding peptide compound of the invention but with more favorable activity than the peptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See, for example, Morgan and Gainor, *Ann. Rep. Med. Chem.*, 24:243-252 (1989). Examples of peptide mimetics are described in U.S. Patent No. 5,643,873. Mimetics of the proteins of the invention may also be made according to other techniques known in the art. For example, by treating a protein of the invention with an agent that chemically alters a side group by converting a hydrogen group to another group such as a hydroxy or amino group.

The invention also includes hybrid genes and peptides, for example where a nucleotide sequence from one species of plant is combined with a nucleotide sequence from another sequence of plant to produce a fusion peptide.

The invention also includes peptide fragments of the proteins of the invention which may be used to confer salt tolerance if the fragments retain biological activity. The invention also includes peptides fragments of the proteins of the invention which may be used as a research tool to characterize the protein or its activity. Such peptides preferably consist of at least 5 amino acids. In preferred embodiments, they may consist of 6 to 10, 11 to 15, 16 to 25, 26 to 50, 51 to 75 or 76 to 100 amino acids of the proteins of the invention (or longer amino acid sequences).

A recombinant nucleic acid molecule for conferring salt tolerance would contain suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, and they may be readily selected by one with ordinary skill in the art. If one were to upregulate the expression of the gene, one would insert the sense sequence and the appropriate promoter into the vehicle. If one were to downregulate the expression of the gene, one would insert the antisense sequence and the appropriate promoter into the vehicle. These techniques are known to those skilled in the art.

Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule. The recombinant molecule may be introduced into the precursor cells or the cells differentiated from the precursor cells using in vitro delivery vehicles such as retroviral vectors, adenoviral vectors, DNA virus vectors and liposomes. Suitable constructs are inserted in an expression vector, which may also include markers for selection of transformed cells. The construct may be inserted at a site created by restriction enzymes. Gene expression levels are controlled with a transcription initiation region that regulates transcription of the gene or gene fragment of interest in a plant, bacterial or yeast cell. The transcription initiation region may be part of the construct or the expression vector. The transcription initiation domain or promoter includes an RNA polymerase binding site and an mRNA initiation site.

Other regulatory regions that may be used include an enhancer domain and a termination region. The regulatory elements described above may be from animal, plant, yeast, bacterial, fungal, viral or other sources, including synthetically produced elements and mutated elements.

In one embodiment of the invention, a plant or yeast cell is transformed with the gene of the invention or a fragment of the gene, inserted in an expression vector to produce a salt tolerant plant. The gene or gene fragment may be either isolated from a native source (in sense or antisense orientations), synthesized, a mutated native or synthetic sequence or a combination of these.

Another embodiment of the invention relates to a method of transforming a plant or yeast cell with the gene of the invention or a fragment of the gene, inserted in an expression vector to produce a salt tolerant plant. The invention also relates to a method of expressing proteins in the plants or yeast cells. The gene of the invention may be used to transform virtually any type of plant, including both monocots and dicots. The plants that may be transformed with the gene of this invention include, but are not limited to the following:

Target plants:

Group I (transformable preferably via *Agrobacterium tumefaciens*)

Arabidopsis
Potato
Tomato
Brassica
Cotton
Sunflower
Strawberries
Spinach
Lettuce
Rice

Group II (transformable preferably via biolistic particle delivery systems (particle bombardment)

Soybean
Rice
Corn

Wheat
Rye
Barley
Atriplex
Salicornia

The gene may also be used with other plants such as oat, barley, hops, sorgum, alfalfa, sunflower, alfalfa, beet, pepper, tobacco, melon, squash, pea, cacao, hemp, coffee plants and grape vines. Trees may also be transformed with the gene. Such trees include, but are not limited to maple, birch, pine, oak and poplar. Decorative flowering plants such as carnations and roses may also be transformed with the gene of the invention. Plants bearing nuts such as peanuts may also be transformed with the salt tolerance gene.

Levels of gene expression may be controlled with genes that code for anti-sense RNA inserted in the expression cassettes or vectors described above.

Transcription is enhanced with promoters known in the art such as the "Super-promoter" [20] or the 35S promoter of cauliflower mosaic virus [21].

Inducible promoters are also used. These include:

a) drought- and ABA-inducible promoters which may include ABA-responsive elements [22,23]

b) heat shock-inducible promoters which may contain HSEs (heat shock elements) as well as CCAAT box sequences [24]

c) salt-inducible promoters which may include AT and PR elements [25]

d) Copper-inducible promoter that includes ACE1 binding sites [26]

e) steroid-inducible promoter that includes the glucocorticoid response element along with an expression vector coding for a mammalian steroid receptor [27].

In addition, tissue specific expression is achieved with the use of tissue-specific promoters such as, the Fd (Ferredoxin) promoter that mediates high levels of expression in green leaves [28] and peroxidase promoter for root-specific expression [29]. These promoters vary in their transcription initiation rate and/or efficiency.

Agrobacterium tumefaciens-mediated transformation, particle-bombardment-mediated transformation, direct uptake, microinjection, coprecipitation and electroporation-mediated gene transfer are used to transfer a salt tolerance gene into plant cells depending upon the plant species. The cells are then used to produce tissue cultures, seeds or whole plants. The methods and compounds for producing mature plants from cells are known in the art.

In a preferred embodiment of the invention, plant tissue cells or cultures which demonstrate salt tolerance are selected and plants which are salt tolerant are regenerated from these cultures. These plants may be reproduced, for example by cross pollination with a plant that is salt tolerant or a plant that is not salt tolerant. If the plants are self-pollinated, homozygous salt tolerant progeny may be identified from the seeds of these plants, for example by growing the seeds in a saline environment, using genetic markers or using an assay for salt tolerance. Seeds obtained from the mature plants resulting from these crossings may be planted, grown to sexual maturity and cross-pollinated or self-pollinated.

The gene is also incorporated in some plant species by breeding methods such as back crossing to create plants homozygous for the salt resistance gene.

A plant line homozygous for the salt tolerance gene may be used as either a male or female parent in a cross with a plant line lacking the salt tolerance gene to produce a hybrid plant line which is uniformly heterozygous for the gene. Crosses between plant lines homozygous for the salt resistance gene are used to generate hybrid seed homozygous for the resistance gene.

The gene of the invention may also be used as a marker in transformation experiments with plants. A salt sensitive plant may be transformed with a salt tolerance gene and a gene of interest which are linked. Plants transformed with the gene of interest will grow beterin a saline environment in which the non-transformed plants are unable to grow.

Example 1

Preparation of polyclonal and monoclonal antibodies.

Hydropathy profiles of the AtNHX6 showed a relatively hydrophilic domain (at the C-terminus) with possible regulatory functions. The C-terminus was sub-cloned into the pGEX - 2TK vector (Pharmacia)) to allow the overexpression of the C-terminus polypeptide as a GST-fusion protein in *E. coli*. The fusion protein was purified by glutathione-affinity chromatography and used as an antigen in rabbits to obtain polyclonal antibodies [30].

Monoclonal antibodies are prepared in mice hybridomas according to established techniques [30] using the C-terminus polypeptide as described above. Polyclonal and monoclonal antibodies raised against other regulatory regions of this Na^+/H^+ antiport are also obtained as described above. The invention includes the antibodies and the hybridoma which secretes the monoclonal antibodies.

Example 2

Identification of similar genes from salt tolerant species.

Several experimental approaches are used to identify similar genes from salt tolerant species. a) We screen cDNA and genomic libraires from sugar beets (a moderate salt-tolerant crop, also known as red beet) under low-stringency conditions with the AtNHX6 Na^+/H^+ antiport cDNA as a probe [31]; b) We apply PCR techniques using degenerate oligonucleotide primers designed according to the conserved regions of the AtNHX6 Na^+/H^+ antiport [32]; c) We screen cDNA expression libraries from different plants (salt-tolerant and salt-sensitive) using antibodies raised against the AtNHX6 Na^+/H^+ antiport [31].

The techniques described above for isolating genes from *Arabidopsis* and sugar beet are used to isolate a salt tolerance gene from Atriplex.

Example 3

Overexpression of the *Arabidopsis* antiport (AtNHX6).

This Na^+/H^+ antiport is constitutively expressed in *Arabidopsis* plants, although the wild type plants are not able to tolerate NaCl concentrations higher than 75 mM. The Na^+/H^+ antiport is overexpressed in these plants in order to improve their tolerance to high salt concentrations. A full length cDNA (coding for the AtNHX6) cloned from an *Arabidopsis thaliana* (Columbia) seedling cDNA library is ligated into a pBISN1 vector [33]. This vector contains a constitutively strong promotor ("super-promotor' [20]. Also, T-DNA vectors (pBECKS) are used [34].Constructs containing the AtNHX6 cDNA with the full Na^+/H^+ antiport open reading frame in a sense orientation were selected by colony hybridization using the AtNHX6 as a probe and by restriction-digest analysis using BglII restriction endonuclease. These constructs are used to transform *Agrobacterium tumefaciens*, and these transformed *Agrobacterium tumefaciens* are used for transformation of *Arabidopsis* plants. The *Agrobacterium* for inoculation is grown at 28°C in a medium containing 5g/l Bacto Beef Extract, 5g/l Bacto-Peptone, 1g/l Bacto Yeast Extract, 240 mg $MgSO_4$ and 5g/l sucrose. The pH will be adjusted to 7.2 with NaOH.

Arabidopsis seeds are washed and surface-sterilized in 5% (w/v) sodium hypochlorite containing 0.15% (v/v) Tween-20. The seeds are rinsed thoroughly with sterile distilled water. Seed aliquots are dispensed in flasks containing 45 ml of cocultivation medium (MS salts, 100 mM sucrose, 10 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l inositol and the pH adjusted to 6.0 with KOH. The flasks are incubated at 22°C under constant rotation (190 rpm) and constant light. After 10-18 h (time needed to break clumps of seeds) 5 ml of log phase of *Agrobacterium* (OD_{600}=0.75) carrying the AtNHX1 construct are added. Twenty-four hours following the inoculation, the seeds are dried by filtration and sown into pre-soaked vermiculite. The flats containing the seeds are irrigated as required with a half-Hoagland solution. The flats are covered with plastic to prevent desiccation and maintained at low artificial illumination. After 3 days the flats are transferred to the greenhouse (the plastic cover removed) under a 16/8 day/night cycle. Supplementary light is provided by high pressure sodium vapor lights. Seven weeks after sowing, the plants are dried

thoroughly and the seeds (T2) harvested. Transformation efficiency is estimated by plating 100,000 seeds (approximately 2.5 g of seeds) on agar plates containing 50 mg/l kanamycin in a medium containing 1% (w/v) sucrose, 0.8 (w/v) agar, MS salts and a pH 6.0 adjusted with KOH. The plates are transferred to a growth room at 25°C under continuous light. After 10 days the kanamycin-resistant seedlings are transferred to new growth medium for 2 weeks and then transferred to small pots containing vermiculite. At senescence (8 weeks) the seeds are collected from single plants (T3). These seeds are germinated and used to assess salt tolerance of the transgenic plants.

Example 4

Overexpression of AtNHX6 in other plants.

Overexpression of AtNHX6 in a number of plants (potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, salicornia, and others) is achieved by *Agrobacterium tumefaciens*-based transformation and/or particle bombardment. The full length cDNA (coding for the AtNHX6) is ligated into the pBINS1 vector or pBECKS (as described above) and these constructs are used to transform *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* used for inoculation is grown as described above. Cultured cells (callus), leaf explants, shoot and root cultures are used as targets for transformation. The targeted tissues are co-cultivated with the bacteria for 1 – 2 days. Afterwards, the tissue is transferred to a growth media containing kanamycin. After one week the tissue is transferred to a regeneration medium containing MS salts, 1% sucrose, 2.5 mg/l 3-benzyladenine, 1 mg/l zeatin, 0.75% agar and kanamycin. Weekly transfers to fresh regeneration media are performed.

A biolistic particle delivery system (particle bombardment) is also used for the overexpression of AtNHX6. Constructs made using a plasmid vector carrying a constitutive promoter, the AtNHX6 open reading frame in a sense orientation and a NOS termination site are used. Plasmid DNA is precipitated into 1.25 mg

of 1-2 μm gold particles using 25 μl of 2.5 M $CaCl_2$ and 10 μl of 0.1 M thiamine (free base). DNA-coated particles are washed with 125 μl of 100% ethanol and then resuspended in 30 μl ethanol. The samples are sonicated to obtain an efficient dispersion, and the samples are aliquoted to obtain delivery disks containing 3 μg of DNA each. Particle bombardment is optimized according to the specific tissue to be transformed. Tissue samples are placed in Petri dishes containing 4.5 g/l basal MS salts, 1 mg/l thiamine, 10 mg/l myoinositol, 30 g/l sucrose, 2.5 mg/l amphotericin and 10 mM K_2HPO_4 at pH 5.7. After bombardment the petri dishes are incubated for 18 – 24 hours. Tissue is regenerated in plates with growth media containing the selective marker. Rooting is initiated and transformed plants are grown under optimal growth conditions in growth chambers. After 2 – 4 weeks the seedlings are transferred to new growth medium for 2 weeks and then transferred to small pots containing vermiculite. At senescence the seeds are collected from single plants. These seeds are germinated and used to assess salt tolerance of the transgenic plants.

Example 5

Overexpression of AtNHX6-homologs in other plants.

Overexpression of AtNHX6-homologs from salt tolerant species (i.e., sugar beet) in other plants (potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, salicornia, and others) is achieved by *Agrobacterium tumefaciens*-based transformation and/or particle bombardment as described above (in Examples 3 and 4). Regeneration of the transformed plants is performed as described in Examples 3 and 4.

Example 6

Expression of AtNHX6, AtNHX6 homologs and AtNHX6 derivatives in *Saccharomyces cerevisiae*.

Expression of AtNHX6, AtNHX6 homologs, and AtNHX6 derivatives in yeast is useful to assess and characterize the biochemical properties of the recombinant and native proteins. Expression in yeast also facilitates the study of interactions between AtNHX6, its homologs and derivatives with regulatory proteins. We have made constitutive expression constructs by ligating the coding region of the AtNHX6 cDNA into a yeast expression vector, pYPGE15, which has a URA3 selectable marker and a PGK promoter. Transformation by lithium acetate [36], 1994), is followed by selection on solid media containing amino acids appropriate for the selection of cells containing the transformation vector. For integrative transformation, the YXplac series of vectors for integrative transformation are used [37].

METHODS

Cloning of the AtNHX6 Arabidopsis Na^+/H^+ antiport cDNA

The genomic DNA sequence of AtNHX6 was identified by us in GenBank on the basis of the similarity of the AtNHX6 translation to the amino acid sequence of AtNHX1 [patent appication, PCT WO9947679A2].
Total RNA was extracted from Arabidopsis thaliana (Ecotype Colombia) seedlings using the Trizol method (LifeTech). 1μg of this RNA was used in RT-PCR reactions as specified in the Ready-To-Go (TM) Kit (AmershamPharmacia Biotech INC). Using the following primers:
X6F3: 5'-CATGAGAGTCAGTTTCTCAC-3'
X6R3: 5'-ATGCTGCCGAAAATGCTGAG-3'
and the two-step protocol described in the manual, a 294 bp fragment was obtained and sequenced. On the basis of the sequence of this fragment, two additional primers were designed to amplify the full-length cDNA by Smart(TM) RACE cDNA Amplification Kit (Clontech):

At6-Ra-3: 5'-GGAGCATTTGGAGCTGATAGGACTCTACATCG-3'
At6-Ra-5: 5'-CAGGCCAGCTCATGAGACCTCTGTGTTCTTTACG-3'
About 1.5 µg total RNA (as above) was used for first-strand cDNA synthesis at 42 degrees Centigrade for 1.5 h, using the primers provided in the Kit. The first strand synthesis reaction was diluted with 100µL Tricine-EDTA. For 5'-RACE, 5 µL of 5'-RACE-Ready cDNA was used as the template. The PCR reaction was conducted under the following temperature cycling conditions: 2 min @ 94°C; 32 cycles of 5 min @ 94°C, 30 seconds @ 65°C, 3 min @ 72°C; an additional 5 min @ 72°C. The primers for 5'-RACE were At6-Ra-5 and the UPM primer provided in the kit. For 3'-RACE, 5 µL of 3'-RACE-Ready cDNA was used as the template. The PCR reaction was conducted under the following temperature cycling conditions: 2 min @ 94°C; 30 cycles of 5 min @ 94°C, 10 seconds @ 68°C, 3 min @ 72°C; an additional 5 min @ 72°C. The primers for 3'-RACE were At6-Ra-3 and the UPM primer provided in the kit. The amplified fragments were recovered and purified following agarose gel electrophoresis. These fragments were then ligated into the pCR2.1-TOPO Vector (Invitrogen) which was then used to transform TOPO10 cells as per instructions in the TOPO TA Cloning Kit (Invitrogen).
The fragments inserted into the vector were sequenced and contigs were assembled to provide the full-legth AtNHX6 cDNA.

Southern Blot Analysis

Genomic DNA was isolated from mature leaf tissue of *Arabidopsis thaliana* (Columbia). 10 µg of this genomic DNA was digested with KpnI, SmaI, XbaI, PstI, BamHI or EcoRI, and fractionated on 0.7% agarose gel, and transferred to Hybond N^+ membrane (Amersham) according to manufacturers instructions. Overnight hybridization was performed at 52°C in Amersham hybridization buffer with a probe labeled with ^{32}P by the random priming method. The template for the probe was the largest fragment of a complete EcoRI digest of the AtNHX6 cDNA. The final wash was in 0.1X SSPE, 0.1% SDS at 50°C.

Hybridization signals were detected by autoradiography on BioMax hyperfilm (Kodak).

Northern Blot Analysis

Arabidopsis thaliana ecotype Columbia was grown either on vertical plates on medium containing 0.5X MS salts and 1% agar at 20-25°C under continuous fluorescent light for 1.5 weeks or in soil at 20-25°C under fluorescent light and incandescent light with a 14 hour photo period for 3-4 weeks. Total RNA was isolated from flower, leaf, and inflorescence stems of the mature plants and from root and shoot tissues of the vertically grown seedlings using TRIZOL reagent (GibcoBRL). 35 µg of RNA was electrophoresed and transferred to Hybond N^+ membrane (Amersham) according to manufacturers instructions. Methylene blue was used to visualize the 26S and 18S ribosomal RNA for quantitation. The blotted RNA was hybridized using the same conditions as for the Southern blot and washed as described for the southern blot analysis.

Yeast Complementation

1. Vector Construction and Transformation

The full length cDNA (coding for AtNHX6) was amplified by PCR with primers:
X_6-YF-5'-GCCGCCCGGGATGACGACTGTAATCGAC-3',
X_6-YR-5'-CCGGCGTCGAC TCATAGATCGTTCCTGAAAACG-3'.
The amplified fragment was gel-extracted and digested by SmaI and SalI, ligated into pYPGE-15 vector which was cut by the same restriction enzymes. Constructs containing the AtNHX6 cDNA with the full-length open reading frame in a sense orientation were selected and verified by sequencing. These constructs were used to transform *Saccharomyces cerevisiae*. Three yeast mutant strains, Δena1-4, Δena1-4 Δnha1 and Δena1-4 Δnhx1 were used in subsequent experiments. Yeast transformation was performed using a conventional lithium acetate method and was followed by selection on solid media containing amino acids appropriate for the selection of cells containing the transformation vector. The transformants were selected on ura^- his^- and ura^- his^-

trp⁻ plates respectively. Colonies from different transformations were selected and grown on APG medium for 2 days at 30 °C with shaking.

2. Yeast Complementation Studies

Three dilutions of each colony culture were spotted on APG plates each with different pH values and NaCl concentrations and grown for 2-4 days at 30°C. Four different pH values of 4.5, 5.5, 6.5 and 7.5 were used. For each pH value the following NaCl concentrations were used: 0, 25mM, 50mM, 75mM, 100mM and 200mM . The Δena1-4 Δnhx1 yeast mutant expressing AtNHX6 showed enhanced growth at pH 4.5 at almost all NaCl concentrations compared to mutant yeast carrying the empty pYGE15 plasmid. At other pH values, there was no significant complemetation was observed. These results demonstrate that the plant AtNHX6 can functionally complement the Δena1-4 Δnhx1 mutation.

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We claim:

1. An isolated nucleic acid molecule for use in preparing a salt tolerant plant or cell, the molecule encoding a PNHX transporter polypeptide, or a fragment of a polypeptide having Na^+/H^+ transporter activity.

2. An isolated nucleic acid molecule for use in preparing a salt tolerant plant or cell, the molecule encoding a TNHX transporter polypeptide, PNHX transporter polypeptide, or a fragment of a polypeptide having Na^+/H^+ transporter activity, comprising a nucleic acid molecule selected from the group consisting of:

   (a) a nucleic acid molecule that hybridizes to all or part of a nucleic acid molecule shown in figure 1, or a complement thereof under moderate or high stringency hybridization conditions, wherein the nucleic acid molecule encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell;

   (b) a nucleic acid molecule degenerate with respect to (a), wherein the nucleic molecule encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

3. The nucleic acid molecule of claim 2, wherein the hybridization conditions comprise moderate or high stringency conditions selected from conditions about those in Table 4.

4. An isolated nucleic acid molecule for use in preparing a salt tolerant plant or cell, the molecule encoding a TNHX transporter polypeptide or a PNHX transporter polypeptide, or a fragment of a polypeptide having Na^+/H^+

transporter activity and capable of increasing salt tolerances in a cell, comprising a nucleic acid molecule selected from the group consisting of:

(a) the nucleic acid molecule of the coding strand shown in figure 1 or a complement thereof;

(b) a nucleic acid molecule encoding the same amino acid sequence as a nucleotide sequence of (a); and

(c) a nucleic acid molecule having at least 17% identity with the nucleotide sequence of (a) and which encodes a TNHX transporter polypeptide or the PNHX transporter polypeptide or a polypeptide having Na+/H+ transporter activity.

5. The nucleic acid molecule of any of claims 1 to 4, wherein the TNHX transporter polypeptide or the PNHX transporter polypeptide comprises an AtNHX transporter polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

6. The nucleic acid molecule of claim 1, comprising all or part of a nucleotide sequence shown in figure 1, or a complement thereof.

7. An AtNHX nucleic acid molecule isolated from *Arabidopsis thaliana*, or a fragment thereof encoding a transporter polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

8. A recombinant nucleic acid molecule comprising a nucleic acid molecule of any of claims 1 to 4 and a constitutive promoter sequence or an inducible promoter sequence, operatively linked so that the promoter enhances transcription of the nucleic acid molecule in a host cell.

9. The nucleic acid molecule of claim 6, wherein the molecule comprises genomic DNA, cDNA or RNA .

10. The nucleic acid molecule of claim 6, wherein the nucleic acid molecule is chemically synthesized.

11. The nucleic acid molecule of claim 6, wherein the nucleic acid molecule is isolated from *Arabidopsis thaliana*.

12. The nucleic acid molecule of any of claims 1 to 4, wherein the TNHX transporter polypeptide or the PNHX transporter polypeptide is capable of extruding monovalent cations out of the cytosol of a cell to provide the cell with increased salt tolerance, wherein the monovalent cations are selected from at least one of the group consisting of sodium, lithium and potassium.

13. The nucleic acid molecule of claim 12, wherein the cell comprises a plant cell.

14. The nucleic acid molecule of claim 13, wherein the monovalent cations are extruded into a vacuole or into the extracellular space.

15. An isolated nucleic acid molecule comprising a nucleic acid molecule selected from the group consisting of 8 to 10 nucleotides of the nucleic acid molecule of claim 6, 11 to 25 nucleotides of the nucleic acid molecule of claim 6 and 26 to 50 nucleotides of the nucleic acid molecule of claim 6.

16. An isolated oligonucleotide comprising at least about 10 nucleotides from a sequence selected from the sequence in figure 1.

17. A vector comprising the nucleic acid molecule of any of claims 1 to 4.

18. The vector of claim 17, comprising a promoter selected from the group consisting of a super promoter, a 35S promoter of cauliflower mosaic virus, a drought-inducible promoter, an ABA-inducible promoter, a heat shock-inducible promoter, a salt-inducible promoter, a copper-inducible promoter, a steroid-inducible promoter and a tissue-specific promoter.

19. A host cell comprising the recombinant nucleic acid molecule of claim 8 or the vector of claim 17, or progeny of the host cell.

20. The host cell of claim 19, selected from the group consisting of a fungal cell, a yeast cell, a bacterial cell, a microorganism cell and a plant cell.

21. A plant, a plant part, a seed, a plant cell or progeny thereof comprising the recombinant nucleic acid molecule of claim 8 or the vector of claim 17.

22. The plant part of claim 21, comprising all or part of a leaf, a flower, a stem, a root or a tuber.

23. The plant, plant part, seed or plant cell of claim 21, wherein the plant, plant part, seed or plant cell is of a species selected from the group consisting of potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, sorgum, alfalfa, salicornia and the plants in Table 5.

24. The plant, plant part, seed or plant cell of claim 21, wherein the plant comprises a dicot plant.

25. The plant, plant part, seed or plant cell of claim 21, wherein the plant comprises a monocot plant.

26. A method for producing a recombinant host cell capable of expressing the nucleic acid molecule of any of claims 1 to 4, the method comprising introducing into the host cell a vector of claim 17.

27. A method of producing a genetically transformed plant which expresses TNHX or PNHX transporter polypeptide, comprising regenerating a genetically transformed plant from the plant cell, seed or plant part of claim 21.

28. The method of claim 27, wherein the genome of the host cell also includes a functional TNHX or PNHX gene.

29. The method of claim 27, wherein the genome of the host cell does not include a functional TNHX or PNHX gene.

30. A transgenic plant produced according to the method of claim 27.

31. A method for expressing a TNHX or PNHX transporter polypeptide in the host cell of claim 19, or the plant, plant part, seed or plant cell of claim 21, the method comprising culturing the host cell under conditions suitable for gene expression.

32. A method for producing a transgenic plant that expresses elevated levels of PNHX transporter polypeptide relative to a non-transgenic plant, comprising transforming a plant with the vector of claim 17.

33. An isolated polypeptide encoded by and/or produced from the nucleic acid molecule of any of claims 1 to 4, or the vector of claim 17.

34. An isolated PNHX transporter polypeptide or a fragment thereof having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

35. The polypeptide of claim 34 comprising an AtNHX transporter polypeptide.

36. The polypeptide of claim 35 comprising all or part of an amino acid sequence in figure 1.

37. A polypeptide fragment of the AtNHX transporter polypeptide of claim 35, or a peptide mimetic of the AtNHX transporter polypeptide, having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

38. The polypeptide fragment of claim 37, consisting of at least 20 amino acids, which fragment has Na^+/H^+ transporter activity and is capable of increasing salt tolerance in a cell.

39. The fragment or peptide mimetic of claim 36, which is capable of being bound by an antibody to the polypeptide of claim 6.

40. The polypeptide of claim 34 which is recombinantly produced.

41. An isolated and purified transporter polypeptide comprising the amino acid sequence of a TNHX transporter polypeptide or a PNHX transporter polypeptide, wherein the transporter polypeptide is encoded by a nucleic acid molecule that hybridizes under moderate or stringent conditions to a nucleic acid molecule in figure 1, a degenerate form thereof or a complement.

42. A polypeptide comprising a sequence having greater than 28% sequence identity to the polypeptide of claim 36.

43. The polypeptide of claim 35, wherein the polypeptide comprises a Na^+/H^+ transporter polypeptide.

44. The polypeptide of claim 43, isolated from *Arabidopsis thaliana*.

45. An isolated nucleic acid molecule encoding the polypeptide of claims 34, 35 or 37.

46. An antibody directed against the polypeptide of claim 36.

47. The antibody of claim 46, comprising a monoclonal antibody or a polyclonal antibody.

Figure 1.

(a)

GTGTTGTTGCTTCTTAGATATATTCAAATAAAATGACGACTGTAATCGACGCGACGATGG
CGTATAGATTTCTGGAGGAAGCGACCGATTCGTCTTCTTCTTCTTCTTCCAAACTAG
AATCTAGCCCTGTCGACGCCGTTCTCTTCGTCGGAATGTCTCTGGTACTCGGTATTGCTT
CTAGGCACTTGCTTCGTGGAACTAGGGTTCCTTACACTGTCGCTCTTCTCGTTATCGGAA
TTGCTCTTGGATCTCTCGAATATGGAGCTAAACATAACCTTGGAAAGATCGGCCATGGAA
TTCGTATCTGGAATGAGATCGATCCAGAACTTCTTTTAGCTGTTTTTCTTCCGGCTCTTC
TTTTCGAGAGTTCGTTTTCAATGGAAGTTCACCAAATTAAGAGGTGTCTGGGACAAATGG
TGTTACTTGCTGTCCCTGGAGTTCTTATTTCAACAGCTTGTCTTGGATCGCTTGTGAAGG
TCACGTTTCCGTATGAATGGGACTGGAAAACGTCCTTGTTGCTTGGGGGACTTTTAAGTG
CTACTGATCCGGTTGCTGTTGTTGCTTTGCTAAAGGAGCTTGGTGCTAGTAAGAAGCTAA
GCACCATAATTGAAGGGGAATCCCTGATGAATGATGGGACGGCGATTGTTGTTTTCCAGT
TATTCTTAAAGATGGCTATGGGGCAAAACTCTGACTGGAGTTCTATAATCAAATTTCTGC
TTAAAGTCGCACTTGGAGCTGTAGGCATTGGTCTGGCGTTTGGCATTGCATCAGTTATTT
GGCTCAAGTTCATATTCAATGACACTGTAATAGAGATTACTCTTACAATTGCAGTGAGCT
ATTTCGCATACTACACTGCTCAAGAGTGGGCTGGGGCTTCTGGTGTTTTGACGGTCATGA
CTTTGGGCATGTTTTATGCTGCATTTGCAAGGACAGCCTTTAAAGGTGACAGTCAAAAAA
GCTTGCATCACTTCTGGGAAATGGTTGCATATATTGCAAACACTTTGATATTTATCCTCA
GTGGTGTTGTCATTGCTGAAGGCATTCTCGACAGTGATAAGATTGCCTACCAAGGGAATT
CATGGCGATTTCTTTTTCTGCTATACGTTTACATCCAACTATCGCGTGTTGTTGTTGTTG
GAGTTCTATATCCACTTTTATGTCGTTTTGGCTATGGTTTGGATTGGAAAGAATCCATTA
TACTCGTATGGTCTGGTTTGAGGGGCGCAGTGGCTCTTGCACTTTCTTTATCCGTGAAGC
AATCAAGCGGAAATTCACATATCAGCAAGGAGACTGGAACATTGTTTCTTTTCTTCACGG
GTGGAATTGTGTTCCTAACTCTGATAGTTAATGGATCCACTACCCAATTTGTTCTACGCC
TTCTTCGCATGGATATTTTACCAGCCCCCAAGAAACGAATATTGGAATATACAAAGTACG
AAATGTTGAATAAGGCCTTACGAGCGTTTCAAGATCTAGGAGACGATGAGGAGCTAGGAC
CTGCTGACTGGCCTACAGTTGAAAGTTATATTTCAAGCCTAAAAGGTTCAGAAGGGGAAC
TAGTTCATCATCCTCACAATGGCTCTAAAATTGGAAGTCTTGACCCTAAAAGTTTAAAGG
ACATACGTATGCGGTTCTTAAATGGTGTGCAAGCAACTTACTGGGAGATGCTTGATGAGG
GCAGAATATCTGAAGTTACTGCTAATATTTTGATGCAGTCAGTGGATGAGGCGCTTGATC
AGGTTTCTACAACTTTATGTGATTGGAGAGGTCTAAAACCACATGTCAATTTCCCAAATT
ACTACAACTTTCTTCATTCTAAAGTTGTCCCACGCAAGTTGGTCACATACTTTGCTGTCG
AAAGACTAGAATCTGCTTGCTACATTTCTGCTGCGTTTCTTCGCGCACATACAATTGTAC
GACAGCAATTGTATGATTTTCTAGGGGAGAGTAATATTGGTTCCATTGTAATCAATGAAA
GTGAAAAGGAAGGAGAGGAAGCAAAAAAGTTCTTGGAAAAAGTCCGATCTTCATTTCCTC
AGGTTCTCCGTGTTGTGAAAACAAAACAAGTAACATATTCAGTGTTGAATCATTTACTCG
GTTACATTGAAAACCACGAGAAGGTTGGCTTGTTGGAGGAAAAAGAAATCGCTCATCTTC
ATGATGCTGTCCAGACCGGCTTGAAAAAGCTTTTGAGAAACCCTCCAATAGTTAAACTTC
CAAAATTGAGCGACATGATCACCTCACATCCGTTATCGGTTGCTCTTCCTCCTGCATTTT
GTGAACCTTTAAAACACTCGAAAAAAGAACCAATGAAACTGCGTGGTGTCACGCTTTATA
AAGAAGGTTCAAAGCCAACTGGAGTCTGGCTTATTTTTGATGGCATCGTTAAGTGGAAAA
GTAAGATCTTAAGCAACAATCACTCGCTGCATCCAACTTTTTCTCACGGTAGTACATTGG
GACTCTACGAAGTCCTCACTGGGAAGCCATATCTGTGCGACTTGATTACAGATTCTATGG
TTCTTTGCTTTTTCATTGATAGCGAGAAAATTCTATCACTACAATCAGATTCTACCATCG
ATGATTTCCTTTGGCAGGAAAGTGCATTGGTTCTTCTCAAACTCTTGCGTCCTCAGATAT
TTGAAAGTGTGGCAATGCAAGAATTACGAGCCCTTGTTTCAACTGAAAGCTCGAAACTTA
CAACATATGTGACGGGAGAATCAATCGAAATCGACTGCAACAGCATTGGTTTATTATTAG
AAGGATTCGTAAAACCGGTTGGTATCAAAGAAGAGCTTATATCATCTCCCGCCGCATTAT
CACCTTCTAACGGGAATCAAAGCTTCCATAATTCATCAGAAGCTTCAGGTATCATGAGAG
TCAGTTTCTCACAACAAGCAACACAGTATATTGTTGAGACGAGAGCAAGAGCAATCATCT

TCAACATTGGAGCATTTGGAGCTGATAGGACTCTACATCGAAGACCATCTTCGTTAACAC
CACCACGTAGCTCAAGCTCTGATCAGCTTCAGAGATCATTTCGTAAAGAACACAGAGGTC
TCATGAGCTGGCCTGAAAATATTTACGCCAAACAACAACAAGAGATCAATAAAACGACAT
TAAGTTTATCTGAACGAGCAATGCAACTCAGCATTTTCGGCAGCATGGTTAATGTGTACA
GAAGGAGTGTAAGTTTCGGTGGGATCTATAATAACAAGTTACAAGATAACTTGTTGTACA
AAAAACTTCCACTAAACCCAGCTCAAGGTCTCGTTTCAGCCAAATCAGAAAGTTCAATTG
TGACCAAGAAGCAGCTTGAAACCCGTAAACATGCGTGTCAGCTTCCTCTGAAAGGGGAAA
GCAGCACAAGGCAAAATACGATGGTTGAATCAAGCGATGAAGAAGATGAAGATGAAGGAA
TCGTTGTGAGAATCGATTCTCCGAGTAAAATCGTTTTCAGGAACGATCTATGAGAATTGA
GATGTTTGTAACATAAGAAAACAAAATTGTTAGCTTACTCTTACAGTTTTACTCATTCAA
ATGTAAAAGCAAAATAATAGTAATAGGCAAAAAAAAAAAAAAAAAAAAAA

(b) amino acid sequence.

MTTVIDATMAYRFLEEATDSSSSSSSSKLESSPVDAVLFVGMSLVLGIASRHLLRGTRVP
YTVALLVIGIALGSLEYGAKHNLGKIGHGIRIWNEIDPELLLAVFLPALLFESSFSMEVH
QIKRCLGQMVLLAVPGVLISTACLGSLVKVTFPYEWDWKTSLLLGGLLSATDPVAVVALL
KELGASKKLSTIIEGESLMNDGTAIVVFQLFLKMAMGQNSDWSSIIKFLLKVALGAVGIG
LAFGIASVIWLKFIFNDTVIEITLTIAVSYFAYYTAQEWAGASGVLTVMTLGMFYAAFAR
TAFKGDSQKSLHHFWEMVAYIANTLIFILSGVVIAEGILDSDKIAYQGNSWRFLFLLYVY
IQLSRVVVVGVLYPLLCRFGYGLDWKESIILVWSGLRGAVALALSLSVKQSSGNSHISKE
TGTLFLFFTGGIVFLTLIVNGSTTQFVLRLLRMDILPAPKKRILEYTKYEMLNKALRAFQ
DLGDDEELGPADWPTVESYISSLKGSEGELVHHPHNGSKIGSLDPKSLKDIRMRFLNGVQ
ATYWEMLDEGRISEVTANILMQSVDEALDQVSTTLCDWRGLKPHVNFPNYYNFLHSKVVP
RKLVTYFAVERLESACYISAAFLRAHTIVRQQLYDFLGESNIGSIVINESEKEGEEAKKF
LEKVRSSFPQVLRVVKTKQVTYSVLNHLLGYIENHEKVGLLEEKEIAHLHDAVQTGLKKL
LRNPPIVKLPKLSDMITSHPLSVALPPAFCEPLKHSKKEPMKLRGVTLYKEGSKPTGVWL
IFDGIVKWKSKILSNNHSLHPTFSHGSTLGLYEVLTGKPYLCDLITDSMVLCFFIDSEKI
LSLQSDSTIDDFLWQESALVLLKLLRPQIFESVAMQELRALVSTESSKLTTYVTGESIEI
DCNSIGLLLEGFVKPVGIKEELISSPAALSPSNGNQSFHNSSEASGIMRVSFSQQATQYI
VETRARAIIFNIGAFGADRTLHRRPSSLTPPRSSSSDQLQRSFRKEHRGLMSWPENIYAK
QQQEINKTTLSLSERAMQLSIFGSMVNVYRRSVSFGGIYNNKLQDNLLYKKLPLNPAQGL
VSAKSESSIVTKKQLETRKHACQLPLKGESSTRQNTMVESSDEEDEDEGIVVRIDSPSKI
VFRNDL

Figure 2. Hydropathy plot of AtNHX6 amino acid sequence. (Kyte and Doolittle hydrophobicity values using a window size of ten amino acids.)

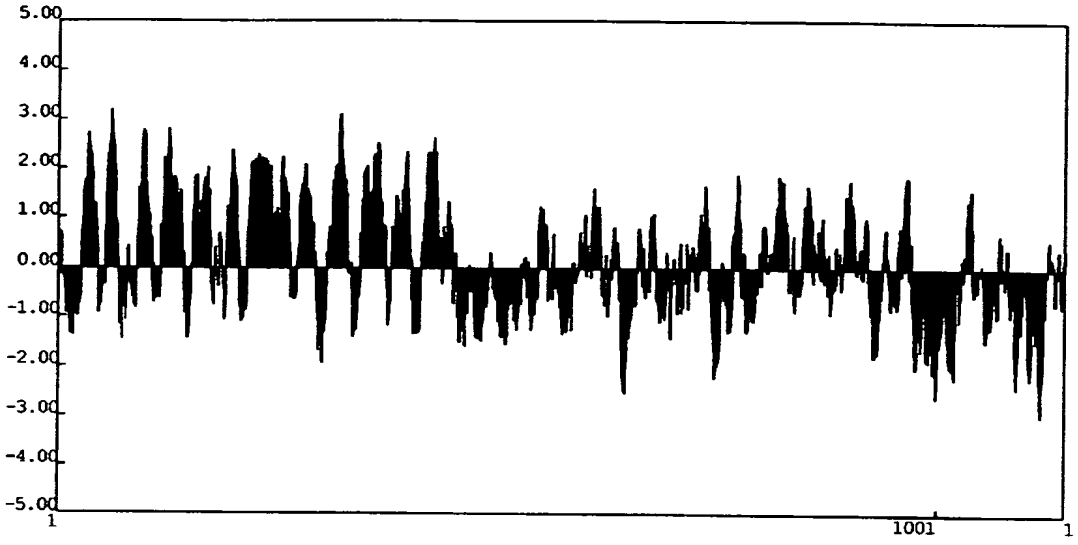
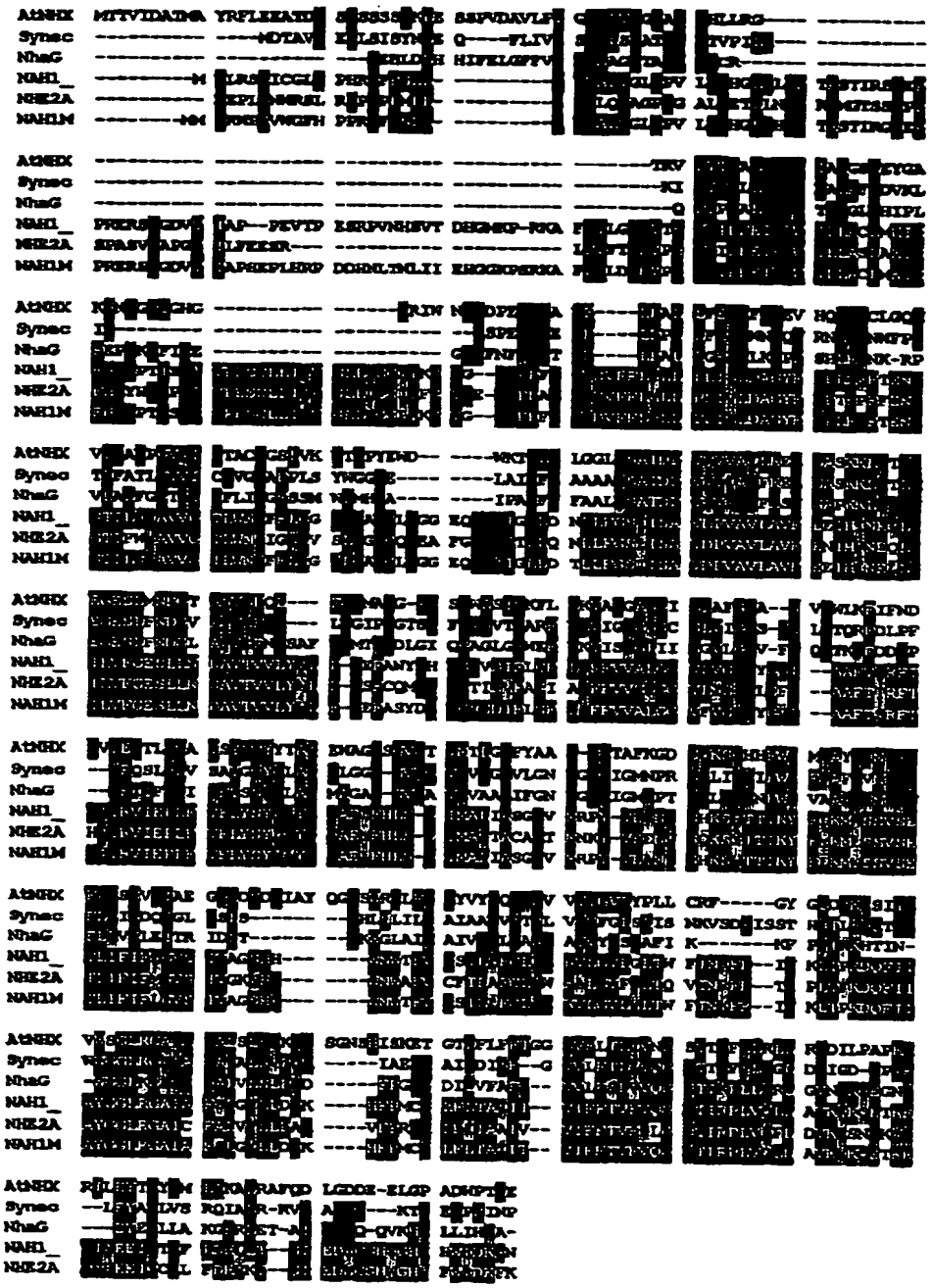


Figure 3. Partial alignment of AtNHX6 with Na^+/H^+ antiports from other organisms.



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(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA PRÉPARATION DES DOSSIERS)